**TITLE:**

Quantification of Antibody-dependent Enhancement of the Zika Virus in Primary Human Cells

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**KEYWORDS:**

Zika virus, dengue virus, pre-immune serum, antibody-dependent enhancement, flaviviruses, viral RNA quantification

**SUMMARY:**

We describe a method to evaluate the effect of pre-existing immunity against dengue virus on the Zika virus infection by using human serum, primary human cells, and infection quantification by quantitative real-time polymerase chain reaction.

**ABSTRACT:**

The recent emergence of the flavivirus Zika and neurological complications, such as Guillain-Barré syndrome and microcephaly in infants, has brought serious public safety concerns. Among the risk factors, antibody-dependent enhancement (ADE) poses the most significant threat, as the recent re-emergence of the Zika virus (ZIKV) is primarily in areas where the population has been exposed and is in a state of pre-immunity to other closely related flaviviruses, especially dengue virus (DENV). Here, we describe a protocol for quantifying the effect of human serum antibodies against DENV on ZIKV infection in primary human cells or cell lines.

**INTRODUCTION:**

Among the mosquitoes-borne viral diseases, Zika infection is one of the most clinically important1. The infection is caused by the flavivirus ZIKV which, in most cases, uses *Aedes aegypti* as its primary vector1,2. However, there are studies that have reported *Aedes albopictus* as a primary vector in some ZIKV outbreaks3. Although the infection is asymptomatic in many cases, the most common symptoms are fever, headache, and muscle pain2. There is no cure or vaccine available for ZIKV infection and the treatment available is mostly supportive. Recent outbreaks of ZIKV in South America led to severe cases of the disease and an approximately 20-fold increase in the neurodevelopmental disorder in fetuses named microcephaly2. As South America is an area endemic to several arboviruses such as DENV and West Nile virus, it is crucial to investigate whether prior immunity to other flavivirus(es) plays a role in the severity of ZIKV infections and disease.

Throughout the ages, viruses have evolved different strategies to increase their chance of infectivity in order to take over the host cell machinery and suppress the antiviral response. One of the most fascinating of all is the use of host pre-immune antibodies by viruses to enhance their replication with the phenomenon ADE4. ADE across all four serotypes of DENV has been well studied and demonstrated to increase viral titers and disease outcome5-7. In a previous *in vitro* study, we have shown significant enhancement of ZIKV replication due to pre-existing DENV immunity in primary human immune cells8. We also demonstrated a relevant *in vitro* method to quantify the capability of DENV pre-existing antibodies to enhance ZIKV replication in primary cells.

The protocol that we have developed uses human serum samples that are tested for DENV neutralization in TCID-50 or plaque reduction neutralization test (PRNT) assays, along with ZIKV in biologically relevant cells or cells derived from tissues that ZIKV can infect.

**PROTOCOL:**

Serum samples used in this study were obtained from human participants of a cohort from Columbia. Sample collection was approved by the internal review board (IRB) at Universidad de Pamplona (Columbia, South America) and Los Potios Hospital8. The samples were anonymously provided and investigators had no access to patient information. The serum samples were checked for the DENV serotype. The samples were further confirmed to neutralize DENV infection *in vitro.* For control, serum samples from healthy individuals (HC) from the USA were used.

NOTE: This protocol can be used to examine the ADE of ZIKV replication in any human cell type expressing the Fcγ receptor. The protocol consists of three parts (**Figure 1**).

1. **Cell Seeding and Infection Setup**

NOTE: For this particular study, human primary macrophages or U937 myelomonocytic cell line (ATCC-CRL-1593.2) were used. The cells were maintained in RPMI growth medium supplemented with 10% fetal bovine serum (FBS) at a 37 °C incubator with 5% carbon dioxide (CO2). All the steps were carried out in a biosafety level 2 (BSL-2) biosafety cabinet in sterile conditions.

1.1. Seed 3 x 104 cells per well in a sterile flat-bottom 96-well plate along with 100 µL of medium and place them in the 37 °C incubator with 5% CO2.

1.2. After seeding the cells, thaw serum samples at room temperature and make 10-fold serial dilutions (*i.e.*, 1/10, 1/100, 1/1,000, 1/10,000) by mixing the samples in serum-free medium. Aliquot the dilutions into a sterile 96-well plate. Use the virus without serum as an additional control.

1.3. Thaw the ZIKV stock at 37 °C in a water bath for 1 - 2 min and quickly transfer them to ice for future use.

1.4. Add a 0.1 multiplicity of infection (MOI) equivalent amount of ZIKV strain MR766 to the serum aliquots.

NOTE: Make sure that the dilution factor remains constant and the total volume is ~200 µL; that is enough for triplicates of each treatment. Keep three wells uninfected to use as negative control for downstream analysis.

1.5. Incubate the serum dilutions with the added virus in the 37 °C incubator with 5% CO2 for 1 h in order to allow the DENV antibodies to form complexes with the Zika virions (for convenience, hereafter referred to as “immune complexes”).

16. Aspirate the media from the cells that were seeded in the flat-bottom 96-well plate. Wash the cells with sterile 1x phosphate-buffered saline (1x PBS).

1.7. Add 50 µL of the immune complexes to each well and incubate them in the 37 °C incubator with 5% CO2 for 2 h.

1.8. After 2 h of incubation, aspirate the media containing the immune complexes from the cells, using a multichannel pipette, and wash the cells 2x with 1x PBS to completely remove the immune complexes and any unattached virions.

1.9. Add 100 µL of fresh complete media supplemented with 10% FBS to each well and incubate the cells in the 37 °C incubator with 5% CO2 for 48 h.

1. **RNA Extraction**

2.1. Remove the 96-well plate from the incubator and transfer it to the biosafety cabinet.

2.2. Aspirate the media, wash the cells 2x with 1x PBS, and proceed to RNA extraction.

NOTE: RNA can be extracted by any method of choice (refer to the **Table of Materials**).

2.3. Add 250 µL of cell lysis buffer with 10% β-mercaptoethanol per well. Pipette the buffer up and down at least 5x along with scratching the cells with the pipette tip to speed up the lysis procedure.

2.4. Transfer the cell lysates to new, labeled, sterile 1.5 mL tubes.

2.5. Add an equal amount of 70% ethanol (250 µL). Pipette up and down 4x - 5x until the mixture is clear.

2.6. Transfer the mixture (~500 µL) to labeled silica-based columns in 2 mL collection tubes and centrifuge at 15,000 x *g* for 30 s. Discard the flow through and keep the columns in the same collection tubes.

2.7. Add 700 µL of wash buffer 1 to each column and centrifuge at 15,000 x *g* for 30 s. Discard the flow through and keep the columns in the same collection tubes.

2.8. Add 500 µL of wash buffer 2 to each column and centrifuge at 15,000 x *g* for 30 s. Discard the supernatant. Repeat this step 2x.

2.9. Transfer the columns to new 2 mL collection tubes and centrifuge at 15,000 x *g* for 2 min. Ensure that silica-based columns get completely dry and there is no ethanol left from wash buffer 2.

2.10. Discard the 2 mL tubes and place the columns in new, sterile, labeled, 1.5 mL recovery tubes.

2.11. Add prewarmed (42 °C) 30 µL of RNase-free water at the center of each column and centrifuge at 15,000 x *g* for 1 min.

2.12. Recover the eluted RNA and quantify the samples, using a spectrophotometer at a 260 nm wavelength.

NOTE: Ideally, determine the purity of the RNA by calculating the ratio between the absorbance values at 260 nm and 280 nm. Purified RNA’s 260/280 ratio is ideally between 1.8 - 2.0.

1. **Quantitative Real-time Polymerase Chain Reaction**

NOTE: Quantitative real-time polymerase chain reaction (qRT-PCR) can be carried out by using any SYBR green mix which usually is composed of SYBR Green I dye, Taq DNA polymerase, deoxynucleotide triphosphates (dNTPs), and a passive dye. Any qPCR machine capable of the detection of SYBR green can be used to perform the reaction and acquire the data. For this experiment, a one-step RT-PCR kit was used which had a cocktail of SYBR Green I, ROX dye, Taq DNA polymerase, dNTPs, and an additional mixture of reverse transcriptase (refer to **Table of Materials**)*.* The primers designed against the envelope region and used in this study to quantify ZIKV genomic levels are CCGCTGCCCAACACAAG for ZIKV-qF and CCACTAACGTTCTTTTGCAGACAT for ZIKV-qR. As a control, human β-2-microglobulin (B2M) was measured and used to normalize the expression of ZIKV (housekeeping gene). The primer sequences to quantify the B2M gene expression used in this study are CTCCGTGGCCTTAGCTGTG for B2M-qF and TTTGGAGTACGCTGGATAGCCT for B2M-qR. Ensure to put three technical replicates for each sample for both the ZIKV and the B2M genes. The setup of the RT-PCR is briefly described below.

**3.1. RT-PCR setup**

3.1.1.Use 100 ng of RNA per sample.

3.1.2.Add 1µL from 10 µM stocks of both forward and reverse primers of a particular gene for each reaction.

3.1.3.Add0.25 µL of reverse transcriptase mix per sample.

3.1.4. For each individual reaction, add 12.5 µL of SYBR Green mix.

3.1.5. Add up to 25 µL of water.Make a master mix of all the above-mentioned reagents except RNA, aliquot it in the 96-well PCR plate, and add RNA last.

3.1.6. Seal the plate with transparent adhesive tape.

3.1.7. Centrifuge the plate at 1,000 x *g* for 1 min to mix the reagents.

3.1.8. Put the plate in the qPCR machine.

**3.2. RT-PCR profile**

NOTE: Use the RT-PCR profile shown in **Table 1**.

3.2.1. Incubate the samples at 50 °C for 10 min in order to ensure the synthesis of complementary DNA (cDNA).

3.2.2. After the cDNA synthesis, activate the Taq DNA polymerase at 95 °C for 5 min.

3.2.3. Perform 40 cycles of denaturation at 95 °C for 10 s and primer annealing and extension at 60 °C for 30 s.

3.2.4. Put the additional melt curve step (65°C) in the end.

**3.3. Data analysis**

3.3.1. Monitor the melt curve by clicking the melt curve tab in the program used to run the qPCR machine, which, ideally, shows a single peak in all the samples for a particular gene to confirm the presence of only one amplicon and an amplification value more than 1.6 if the algorithm considers 2 as the 100% amplification value (the amplification value varies according to the algorithm used by the qPCR machine). Make sure to use 0.5 °C temperature increments between steps and a minimum holding time of 10 s in the melt curve protocol, for optimal results.

3.3.2. After making sure there are a single amplicon and good amplification value, first click on the quantification data tab to get a quantitative cycle (Ct/Cq value) of each sample and export to Microsoft Excel.

3.3.3. Using a spreadsheet, calculate the average of each sample using the Ct value. Next, click in the formulas and select the average. The average CT values of each sample (replicate) are used for further analysis.

3.3.4. Next, calculate the ΔCt using the formula ΔCt = average Ct of the target gene – average Ct of the control gene (in this case, ΔCt = average Ct of the ZIKV gene – average Ct of the B2M gene).

3.3.5. Calculate ΔΔCt to normalize the data (in this case, ΔΔCt = ΔCt ZIKV samples – B2M samples).

3.3.6. Calculate the fold increase gene expression by typing the formula 2^-(ΔΔCt) for every single ΔΔCt normalized value. The results of the fold increase to perform statistical tests can be obtained as described in earlier studies9-10.

**REPRESENTATIVE RESULTS:**

In **Figure 1**, there is a step-by-step diagrammatic illustration of all the steps involved to carry out the ADE protocol. It is a schematic diagram showing the whole procedure of ADE of ZIKV due to pre-existing immunity to DENV. **Figure 2** shows how human serum samples were categorized into three different groups: DENV infection-confirmed samples are referred to as the DENV-infected group, DENV antibody-confirmed samples are referred to as the DENV-exposed group, and healthy individuals’ sera with no DENV-neutralizing antibodies or RNA are called the healthy control (HC) group. (**Figure 2**).

All the serum samples were allowed to make complexes with ZIKV and were then used to infect macrophage cells. After 48 h postinfection, RNA was extracted and subjected to qRT-PCR. The representative experiment in **Figure 3** demonstrates that most of the sera containing DENV serotype 1 to 4 antibodies were able to enhance ZIKV replication at different levels. The highest increase in ZIKV titers was found in the macrophages treated with sera containing DENV serotype 2 and 4 antibodies as compared to serotype 1 and 3, which showed a relatively less induction of ZIKV.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Diagrammatic illustration of the ADE protocol.** A step-by-step illustration shows all the steps involved in the whole protocol.

**Figure 2: Schematic of the experimental procedure.** Three types of sera were incubated with Zika virus. Group I consisted of DENV-RNA positive (DENV-infected) samples. Group II consisted of DENV-antibody positive (DENV-exposed) samples. Group III consisted of samples with no DENV RNA or antibody (healthy controls). The virus-sera mixture was added to human macrophages and the infection quantified. This figure has been modified from Londono-Renteria *et al.*8.

**Figure 3: DENV immune sera enhances ZIKV infection in primary human macrophages.** Human sera containing DENV antibodies (DENV1-4 are serotype confirmed, Col are serotype unknown) or from healthy controls were diluted 1:10 to 1:10,000 and incubated with ZIKV. The sera are described in **Table 1**. Primary isolated human macrophages were infected either with ZIKV alone or with the ZIKV-sera mixtures. (**a**) DENV1 antibody-containing sera. (**b**) DENV2 antibody-containing sera. (**c**) DENV3 antibody-containing sera. (**d**) DENV4 antibody-containing sera. (**e**) DENV-antibody sera from Colombian individual 1. (**f**) DENV-antibody sera from Colombian individual 2. The infection was measured by qRT-PCR analysis at 48 h postinfection. Technical and biological replicates were done in triplicate. The data are pooled, and the error bars indicate standard deviation. Student’s *t*-test and ANOVA were used for the statistical analysis. \* *P* < 0.001. This figure has been modified from Londono-Renteria *et al.*8.

**Table 1: Thermal profile of qRT-PCR.**

**DISCUSSION:**

Cross-reactivity of DENV antibodies leading to the ADE of other DENV serotypes has hindered the development of an effective vaccine11. ZIKV belongs to the same family, Flaviviridae, and has a considerable homology with other flaviviruses, especially DENV12. The main target of neutralizing antibodies for both ZIKV and DENV is the envelope protein, which shares a very high structural and quaternary sequence homology between the two viruses13-15. It has been demonstrated that pre-immunity to either DENV or ZIKV can enhance the infection of the other virus8,16.

There are a number of different methods employed to quantify the viral infectivity in ADE experiments, ranging from plaque assays17, intracellular viral antigen staining using antibodies conjugated with fluorescent dyes, and flow cytometry18,19. These assays are time-consuming and not easily adaptable for the high-throughput of multiple samples at the same time. Importantly, most of the studies used DENV-specific monoclonal antibodies to check their effect on ZIKV and examined the ADE in cell lines only20,21. In this protocol, we describe a simple yet effective method in which we used human pre-immune serum samples that have a neutralizing ability against DENV, along with primary human immune cells, to examine the effect of patient serum on ZIKV replication by employing qRT-PCR. This method is robust, relevant, and can be completed in three days. Although the representative results in this manuscript show its application for ZIKV, this protocol can be easily modified and used for other flaviviruses, like yellow fever virus, dengue virus, and West Nile virus. One important factor to consider is that this protocol has a limitation in distinguishing between mature and immature viral RNA.

During the protocol, it is critical to give careful consideration when performing the RNA extractions, ensuring that the environment is RNase-free during all the RNA handling steps. Furthermore, viral stocks should be thawed at 37 °C in a water bath for 1 - 2 min and immediately put on ice. However, the virus should not be kept on ice for long as, then, it can lose its infectivity.

Previous results have demonstrated that this protocol is highly convenient and adaptable to handle a large number of samples and can be completed in relatively less time than other methods. This protocol has the potential to be used as a useful assay for future ADE studies.

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**DISCLOSURES:**

The authors have nothing to declare.

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